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Genomic and cDNA Cloning, Characterization of *Delonix regia* Trypsin Inhibitor (DrTI) Gene, and Expression of DrTI in *Escherichia coli*

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Degenerate primers were designed based on all possible sequences of the N-terminal and C-terminal regions of *Delonix regia* trypsin inhibitor (DrTI). Five hundred sixty-one bp of polymerase chain reaction (PCR) product was amplified using the above degenerate primers and genomic DNA and cDNA of *Delonix regia* as a template. The amplified PCR products were cloned and sequenced. DNA sequence analysis of cDNA and genomic clones of DrTI have the same nucleotide sequence in the coding region, and manifested a genomic clone without intervening sequences in the coding region. The amino acid sequence deduced from the DrTI genomic and cDNA clones agreed with that identified *via* amino acid sequencing analysis, except that two amino acid residues, Ser and Lys, existed between residues Lys141 and Ser142. DrTI open reading frame was then amplified and cloned in-frame with GST in pGEX4T-1 and overexpressed in *Escherichia coli* to yield a glutathione S-transferase (GST)-fusion protein with a calculated molecular mass of about 45 kDa. The recombinant DrTI (reDrTI) was derived by treating the GST-DrTI fusion protein with thrombin. Both the reDrTI and GST-DrTI fusion protein exhibited a strong identical inhibitory effect on trypsin activity.

Key words: *Delonix regia* trypsin inhibitor; Kunitz-type trypsin inhibitor; *Delonix regia*; molecular cloning

Seed proteins have important roles in plant survival such as maintaining seed viability, providing nutrition during early seeding, and protecting the seeds against microbes and insects.[1] Protease inhibitors of seeds can inhibit trypsin while it passes through the gut of an animal, thus helping with seed dispersal, and protecting plants against pests and diseases.[2–5] Protease inhibitors are present in significant quantities in *Leguminosae* seeds and in smaller quantities in cereals, cucurbits, potatoes and other tubers.[6–8] Numerous studies have recently demonstrated the efficacy of proteinase inhibitors as defense proteins; the most direct proof comes from proteinase inhibitor overexpression in transgenic plants, which causes increases in resistance to insect pests.[9–11] Besides their natural biological functions, proteinase inhibitors might also be useful in treating human pathologies such as inflammation, hemorrhage,[12] and cancer.[13–16]

Serine proteinase inhibitors from plants are classified into families: the Kunitz trypsin, Bowman-Birk proteinase, potato I, potato II, barley trypsin, and squash inhibitor families.[17] The legume proteinase inhibitors are further classified into two main groups according to their size and cysteine content. Kunitz-type inhibitors are proteins (Mr 18,000–22,000) with one or two polypeptide chains and low cysteine content, generally with four cysteine residues arranged into two disulfide bridges, each comprising 170–180 amino acids. *Delonix regia* trypsin inhibitor (DrTI) which belongs to the Kunitz family, is purified from *Delonix regia* (Leguminosae Caesalpinioideae) seeds. The primary structure of DrTI has been identified.[18] It comprises a single-polypeptide chain with a molecular mass of 22 kDa and two disulfide bonds. The amino acid sequence of DrTI has a high similar comparative sequence of related Kunitz inhibitors, including SBTI,[19] SwTI,[20] PtTI,[21] EcTI,[22] BvTI-3c and ACTI.[23,24] Finally, DrTI is an effective inhibitor of trypsin and human plasma kallikrein, but not of chymotrypsin, plasmin, factor Xa or tissue kallikrein.

Sequence comparison with other plant trypsin inhib-

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Abbreviations: DrTI, *Delonix regia* trypsin inhibitor; L-BAPNA, α-N-benzoyl-D,L-arginine-4-nitro-anilide hydrochloride; re, recombinant; GST, glutathione S-transferase; SDS–PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; DTT, dithiothreitol; PCR, polymerase chain reaction; ORF, open reading frame itors of the Kunitz family reveals that, instead of the conserved Arg or Lys found in other Kunitz TIs, DrTI contains a negatively charged residue (Glu68) at the P1 reactive site.[18] The present study aimed to clone the cDNA and genomic DNA of the DrTI gene and to express GST-DrTI fusion protein and reDrTI which exhibit a strong identical inhibitory effect on trypsin activity (*E. coli)*. In the future, mutant proteins can perhaps be utilized by site-directed mutagenesis to investigate the role of Glu68 residue in trypsin inhibitory activity of DrTI.

Materials and Methods

Materials. Isopropyl-1-thio-D-galactopyranoside (IPTG), PCR marker, T4 DNA ligase and thrombin were purchased from Promega (Madison, WI). The expression vector, pGEX4T-1, and a HiTrap™ DEAE FF column were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Trypsin, glutathione-agarose gel and L-BAPNA were from Sigma (St. Louis, MO). Restriction enzymes and other reagents used in molecular-biology techniques were purchased from New England Biolabs, Boston, MA. A DNeasy Plant Maxi kit and an Oligotex mRNA Purification System were from Qiagen (Hilden, Germany). All other chemicals used were of analytical grade.

Isolation of Delonix regia *genomic DNA and mRNA, and cDNA synthesis. Delonix regia* genomic DNA was extracted from 1 g of lyophilized young leaves using a silica-gel membrane-type kit (DNeasy Plant Maxi, Qiagen, Hilden, Germany) based on a previous study.[25] Maturing *Delonix regia* seeds about one month after flowering were obtained from a local source (Hsinchu, Taiwan). Total cellular RNA was isolated from the seeds by homogenizing them in 4 M guanidinium thiocyanate.[26] Poly $(A)^+$ was purified from total cellular RNA using the Oligotex mRNA Purification System. Poly $(A)^+$ rich RNA from the seeds of *Delonix regia* was used for cDNA synthesis as described previously.[27] The genomic DNA and cDNA samples were used for the subsequent PCR analysis.

Amplification of Delonix regia *genomic DNA and cDNA with DrTI specific primers.* On the basis of the amino acid sequence of DrTI,[15] two degenerate PCR primers were prepared. Primer A_{start} (5′-TCNGAYGCN-GARAARGTNTAYGAYATHGA-3′) encodes the first eight N-terminal amino acids of the DrTI. Primer A_{stop} (5′-NGAYTCNGTYTCNGANCCNGANCGNGGYTT-3′) encodes the last eight C-terminal amino acids of the DrTI. The reaction mixture for PCR was prepared in a PCR reaction tube. The reaction volume of 50 µl contained 1 × PCR buffer (20 mM Tris–HCl, pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton-X-100 and 5 µg BSA), 500 ng of genomic DNA or 500 ng cDNA, 400 µM dNTP, 1.5 mM $MgCl_2$, 50 pmole of each of paired A_{start}/A_{stop} primers and 2.5 units *Pfu* DNA polymerase (Promega, Madison, WI). The reactions were amplified in a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) according to the following PCR step-cycle program: pre-incubation at 95 °C for 5 min, denaturation at 95 °C for 1 min, annealing at 45 °C for 1 min, and extension at 72 °C for 2 min. The cycle was repeated 35 times followed by a final extension at 72 °C for 7 min. Reaction products were analyzed by electrophoresis on 1.5% agarose gel in Tris/acetate EDTA (40 mM Tris–HCl, pH 8.0, 5 mM sodium acetate, 1 mM EDTA). The products were detected by ethidium bromide staining.

Construction of genomic and cDNA clones of DrTI. DNA fragments of 0.56 kbp isolated and produced by PCR reaction by agarose gel electrophoresis were ligated to pBluescript vector (Stratagene, La Jolla, CA) which was digested Sma I and calf intestine phosphatase. Transformed *E. coli* cells (TG 1) were selected by blue-white selection. Clones with 561 bp fragments were sequenced using an ABI 310 automated sequencer. All inserts were sequenced at least twice on both strands.

Construction of expression plasmids and overexpression of GST-DrTI fusion proteins in E. coli. Expression plasmid was the derivative of pGEX-4T-1 and was constructed by ligating a 561 bp BamHI/EcoRI fragment derived from pcDrTI by PCR with primer-A, 5′ AAGGATCCTCGGACGCGGAGAAGGTTT 3′ and primer-B, 5′ AGGAATTCTTAGGACTCCGTTTCCGAT 3′,[28] which contained the entire DrTI coding sequence in-frame into the BamHI/EcoRI sites of pGEX4T-1. The resulting construct, pGEX4T-1-cDrTI contained both glutathione S-transferase and the DrTI gene. All the constructs of DrTI cDNA were confirmed by sequencing the ligation products of pGEX4T-1 plasmids. To enlarge the expression of DrTI fusion protein, *E. coli* TG 1 cells harboring pGEX4T-1-cDrTI construct were grown at 37 °C in 500 ml of LB broth (1% NaCl, 1% Bacto-tryptone, 0.5% Bacto-yeast extract, pH 7.0) containing 100 µg/ml of ampicillin. When OD_{600} reached 0.6, IPTG was added to a final concentration of 1 mM to induce fusion protein expression and the culture was incubated for a further 4 h at 30 °C. A maximal harvest was obtained under these conditions. Total soluble proteins were extracted in resuspended buffer (10 mM Na_2HPO_4, 1.8 mM NaH_2PO_4, 140 mM NaCl, 2.7 mM KCl, pH 7.5, 1 mM DTT, 0.2 mg/ml lysozyme) by repeated freeze-thawing, followed by centrifugation (10,000 × *g*, 10 min) at 4 °C, the supernatant being the crude protein extract. Fifteen µg of crude protein extract was analyzed on 12.5% SDS–PAGE followed by Coomassie blue staining.

Purification of GST-DrTI fusion proteins and reDrTI. GST-DrTI fusion proteins were produced and purified to homogeneity as described previously.[29] In brief, crude protein extracts were loaded onto a glutathione-agarose affinity column, and then, after washing, the GST-DrTI fusion protein was eluted using 5 mM reduced glutathione in 50 mM Tris–HCl, pH 8.0. The fusion proteins were then treated with thrombin to liberate the recombinant DrTIs which were purified with a HiTrap™ DEAE FF column (1 ml). After applying the sample to the column, it was eluted with 50 mM Tris/HCl, pH 8.0, and then eluted with a linear gradient of 0 to 0.3 M NaCl in the buffer.

Trypsin Inhibitory activity of reDrTI. The trypsin inhibitory activities of GST-DrTI fusion protein, reDrTI and native DrTI were measured by incubating each DrTI with trypsin in 1 ml of 0.1 M Tris–HCl buffer, pH 8.0 containing 0.01 M $CaCl_2$ for 5 min at 37 °C. Residual trypsin activity was determined by adding 10 ml L-BAPNA (50 mg/ml in DMSO) at 37 °C. After 20 min of incubation, the reaction was stopped by adding 0.5 ml of 10% acetic acid. The degree of inhibition was determined by measuring the optical density at 410 nm.[30]

Results and Discussion

Amplification and analysis of DrTI cDNA and genomic fragment

The application of degenerate primers based on the amino acid sequence for DrTI was used to amplify the specific sequences of DrTI cDNA and genomic DNA, and the expected size obtained was as illustrated in (Fig. 1). The main product obtained following amplification of DrTI cDNA and genomic DNA with primers A_{start}/A_{stop} annealed at 45 °C was a fragment of approximately 0.56 kbp.

The amplified DNA fragments produced by PCR reaction with cDNA and genomic DNA as template were subsequently subcloned to pBluescript vector and sequenced. Analysis of cloned DNA fragments from PCR reaction using either cDNA or genomic DNA as template revealed cloned DNA of the expected size (0.56 kbp) covering the entire DrTI. Random sampling and sequencing of clones containing the 561 bp fragment obtained detected only one type, and (Fig. 2) showed that the deduced amino acid sequence of DrTI using DNA sequence analysis of cDNA and genomic clones of DrTI had the same nucleotide sequence in the coding region, and identified the genomic clone without intervening sequences in the coding region. The amino acid sequence deduced from the cDNA clone agreed with that determined by amino acid sequencing analysis, except for two amino acid residues, Ser and Lys, between residues Lys141 and Ser142 of the amino acid sequence.[18] The genomic clones isolated from soybeans encoding kunitz-type trypsin inhibitors and Bowman-Birk-type trypsin inhibitor have been reported not to contain an intron.[30–32]

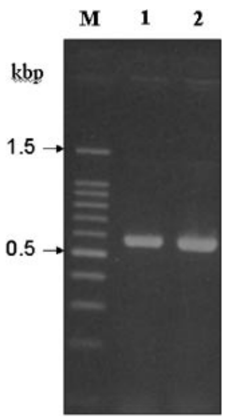

Fig. 1. Amplification of *Delonix regia* Gemonic DNA and cDNA Using DrTI Degenerate Primer Pairs A_{start}/A_{stop}.

Lane M, 100 bp ladder marker. Lane 1, PCR products obtained with cDNA as a template. Lane 2, PCR products obtained with genomic DNA as a template. Five µl of PCR reaction was applied to the agarose gel, and the gel was stained with ethidium bromide.

Construction of expression plasmids and overexpression of GST-DrTI fusion proteins in E. coli

The primers were applied to PCR on the pcDrTI to generate DrTI-encoding DNA fragments for subcloning. DNA fragments encoding 187 amino acids of DrTI flanked by EcoRI and BamHI were ligated into pGEX4T-1 using T4 DNA ligase. The desired expression vector with insert was confirmed *via* nucleotide sequencing. The expression plasmid was designated pGAEX4T-1-cDrTI.

The GST-DrTI fusion proteins were obtained from pGEX4T-1-cDrTI expression in *E. coli* TG1 cells. The fusion proteins were purified from the *E. coli* lysate through affinity chromatography with a glutathione-agarose gel column. The purified fusion proteins were then treated with thrombin and purified with a HiTrap™ DEAE FF column. This study obtained a final yield of 5–6 mg purified reDrTI/liter induced *E. coli* at 30 °C. The homogeneity of purified reDrTIs was analyzed using SDS–PAGE and the results are shown in (Fig. 3). The N-terminal amino acid sequence of reDrTIs was determined using an automatic sequencer, and the results revealed that reDrTI has the same N-terminal amino acid sequence (about 15 amino acid residues) as native DrTI, except for two extra amino acid Gly-Ser at the N-terminus (data not shown).

Trypsin inhibitory activity of GST-DrTI fusion protein and reDrTI

The trypsin inhibitory activity of the fusion protein and reDrTI was tested and compared with that of native DrTI (Fig. 4). On a molar basis, the reDrTI and fusion protein exhibited the same 50% inhibition concentration as the native DrTI. The Ki value of the reDrTI was 21.9 nM, the same as that of native DrTI.[18]

General discussion

To clone the DrTI gene, PCR amplification was

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1    TCGGACGCGGAGAAGGTTTACGACATAGAAGGCTACCCTGTATTCTTAGGTTCTGAATACTACATAGTGTCAGCTATCATTGGGGCTGGT
1     S  D  A  E  K  V  Y  D  I  E  G  Y  P  V  F  L  G  S  E  Y  Y  I  V  S  A  I  I  G  A  G
91   GGTGGTGGGGTTAGACCTGGGAGAACTCGGGGCTCCATGTGCCCAATGTCTATCATCCAAGAACAATCTGATCTTCAAATGGGTCTCCCA
31    G  G  G  V  R  P  G  R  T  R  G  S  M  C  P  M  S  I  I  Q  E  Q  S  D  L  Q  M  G  L  P
181  GTAAGATTCAGTAGTCCAGAAGAAAGCCAAGGCAAAATATATACCGACACTGAACTGGAAATAGAGTTCGTGGAGAAGCCAGACTGTGCA
61    V  R  F  S  S  P  E  E  S  Q  G  K  I  Y  T  D  T  E  L  E  I  E  F  V  E  K  P  D  C  A
271  GAATCTTCCAAGTGGGTGATCGTTAAGGACTCAGGCGAAGCAAGGGTGGCTATAGGTGGCTCTGAGGACCATCCACAAGGCGAGCTGGTA
91    E  S  S  K  W  V  I  V  K  D  S  G  E  A  R  V  A  I  G  G  S  E  D  H  P  Q  G  E  L  V
361  AGGGGGTTTTTCAAGATTGAGAAACTTGGATCACTCGCCTACAAGCTTGTGTTTTGTCCCAAAAGCAAATCCTCTTCAGGTAGTTGTTCA
121   R  G  F  F  K  I  E  K  L  G  S  L  A  Y  K  L  V  F  C  P  K [S  K] S  S  S  G  S  C  S
451  GATATTGGGATTAATTATGAGGGCAGAAGGAGTCTGGTTCTGAAAAGTAGTGATGACTCGCCATTCCGTGTCGTGTTCGTAAAACCCCGG
151   D  I  G  I  N  Y  E  G  R  R  S  L  V  L  K  S  S  D  D  S  P  F  R  V  V  F  V  K  P  R
541  TCAGGATCGGAAACGGAGTCC
181   S  G  S  E  T  E  S
```

Fig. 2. Nucleotide Sequence of DrTI cDNA in the Coding Region and Its Deduced Amino Acid Sequence.

Locations of oligonucleotide primers used in primer extension analyses are shown by underlining. The box indicates the amino acids with different sequence compared by amino acid sequence analysis.[18)] The GenBank accession numbers of the nucleotide sequences of DrTI cDNA and genomic DNA in the coding region are DQ019824 and DQ019825, respectively.

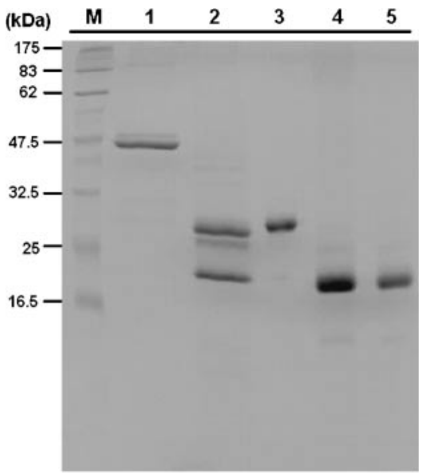

Fig. 3. Twelve Percent SDS–PAGE Analysis of the Fusion Protein, reDrTI and Native DrTI.

Lane M, molecular-mass markers. Lane 1, GST-DrTI fusion protein. Lane 2, fusion protein treated with thrombin. Lane 3, glutathione S-transferase. Lane 4, reDrTI. Lane 5, native DrTI.

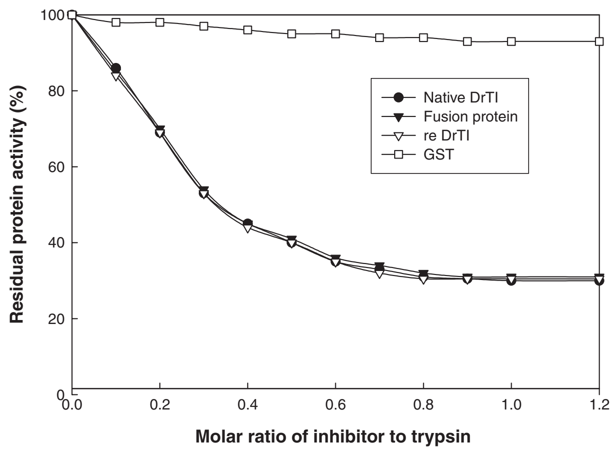

Fig. 4. Trypsin Inhibitor Activity of GST-DrTI Fusion Protein, reDrTI and Native DrTI.

Increasing concentrations of inhibitor with respect were to a fixed concentration of trypsin (2 nM). Residual enzyme activity was determined by using L-BAPNA as substrate. Native DrTI was purified from *Delonix regia* seed as described elsewhere.[18)] Each point is the mean of three assays.

performed using *Delonix regia* genomic DNA isolated from young leaves or cDNA isolated from maturing seeds as a template and the same degenerate PCR primers based on all possible sequences of the N-terminal and C-terminal regions of DrTI. These amplified products had the same size as the genomic DNA and cDNA. DNA sequence analysis of cDNA and genomic clones of DrTI displayed the same nucleotide sequence in the coding region. The correct nucleotide sequence of the primer parts of DrTI was not observed in cDNA or genomic clones because the DrTI clone included degenerate primers. This result shows that the DrTI gene did not contain intervening sequences in the coding region. The amino acid sequence deduced from the cDNA clone agreed with that determined by amino acid sequencing analysis, excepts for the existence two amino acid residues, Ser and Lys, between residues Lys141 and Ser142 of the amino acid sequence. The difference in amino acid sequence between the cDNA and the protein of DrTI indicates that *Delonix regia* contains several related trypsin inhibitor genes as soybean (Gly*cine mnx*) trypsin inhibitors.[30,33)]

This study obtained a final yield of 5–6 mg of purified reDrTI/liter-induced *E. coli* at 30 °C. The reDrTI and fusion protein had the same 50% inhibition concentration as the native DrTI. The Ki value of the reDrTI was determined to be 21.9 nM, the same as that of native DrTI.

An interesting peculiarity of DrTI is that a Glu68 residue was found in the expected position for the reactive site rather than an Arg or Lys, usually present in other Kunitz-type inhibitors.[18] *Swartzia pickellii* trypsin inhibitor also contains a Glu residue in the reactive site for trypsin.[20] In the future, mutant proteins can perhaps be utilized by site-directed mutagenesis to investigate the role of Glu68 residue in trypsin inhibitory activity of DrTI.

Acknowledgments

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References

1) Millerd, A., Biochemistry of legume seed proteins. *Annu. Rev. Plant Physiol.*, **26**, 53–72 (1975).
2) Ryan, C. A., Protease inhibitor gene families: strategies for transformation to improve plant defenses against herbivores. *BioEssays*, **10**, 20–24 (1989).
3) Broadway, R. M., Are insects resistant to plant proteinase inhibitors? *J. Insect Physiol.*, **41**, 107–116 (1995).
4) Koiwa, H., Bressan, R. A., and Hasegawa, P. M., Regulation of protease inhibitors and plant defense. *Trends Plant Sci.*, **2**, 379–384 (1997).
5) Ryan, C. A., Proteolytic enzymes and their inhibitors in plants. *Annu. Rev. Plant Physiol.*, **24**, 173–196 (1973).
6) Richardson, M., Seed storage proteins: the enzyme inhibitors. In "Methods in Plant Biochemistry," eds. Dey, P. M., and Harborne, J. B., Academic Press, New York, pp. 259–305 (1991).
7) Yeh, K. W., Chen, J. C., Lin, M. I., Chen, Y. M., and Lin, C. Y., Functional activity of sporamin from sweet potato (*Ipomoea batatas* Lam.): a tuber storage protein with trypsin inhibitory activity. *Plant Mol. Biol.*, **33**, 565–570 (1997).
8) Hou, W. C., Lin, J. S., Chen, H. J., Chen, T. E., Chang, C. F., and Lin, Y. R., Dioscorin, the major tuber storage protein of yam (*Dioscorea batatas decne*) with carbonic anhydrase and trypsin inhibitor activities. *J. Agric. Food Chem.*, **47**, 2168–2172 (1999).
9) Schuler, T. H., Poppy, G. M., Kerry, B. R., and Denholm, I., Insect-resistant transgenic plants. *Trends Biotechnol.*, **16**, 168–175 (1998).
10) Heitz, T., Geoffroy, P., Fritig, B., and Legrand, M., The PR-6 family: proteinase inhibitors in plant-microbe and plant insect interactions. In "Pathogenesis-Related Proteins in Plants," eds. Datta, S. K., and Muthukrishan, S., CRC Press, Boca Raton, pp. 131–155 (1999).
11) Lee, S. I., Lee, S. H., Koo, J. C., Chun, H. J., Lim, C. O., Mun, J. H., Song, Y. H., and Cho, M. J., Soybean Kunitz trypsin inhibitor (SKTI) confers resistance to the brown planthopper (*Nilaparvata lugens* Stal) in transgenic rice. *Mol. Breed*, **5**, 1–9 (1999).
12) Oliva, M. L. V., Souza-Pinto, J. C., Batista, I. F. C., Araujo, M. S., Silveira, V. F., Auerswald, E. A., Mentele, R., Eckerskorn, C., Sampaio, M. U., and Sampaio, C. A. M., *Leucaena leucocephala* serine proteinase inhibitor: primary structure and action on blood coagulation, kinin release and rat paw edema. *Biochim. Biophys. Acta*, **1477**, 64–74 (2000).
13) DeClerck, Y. A., Imren, S., Montgomery, A. M., Mueller, B. M., Reisfeld, R. A., and Laug, W. E., Proteases and protease inhibitors in tumor progression. *Adv. Exp. Med. Biol.*, **425**, 89–97 (1997).
14) Kennedy, A. R., The Bowman-Birk inhibitor from soybeans as an anticarcinogenic agent. *Am. J. Clin. Nutr.*, **68**, 1406S–1412S (1998).
15) Lin, J. Y., Hsieh, Y. S., and Chu, S. C., Chimeric protein: abrin B chain-trypsin inhibitor conjugate as a new antitumor agent. *Biochem. Int.*, **19**, 313–323 (1989).
16) Armstrong, W. B., Kennedy, A. R., Wan, X. S., Taylor, T. H., Nguyen, Q. A., Jensen, J., Thompson, W., Lagerberg, W., and Meyskens, F. L. Jr., Clinical modulation of oral leukoplakia and protease activity by Bowman-Birk inhibitor concentrate in a phase IIa chemoprevention trial. *Clin. Cancer Res.*, **6**, 4684–4691 (2000).
17) Richardson, M., "Methods in Plant Biochemistry" Vol. 5, eds. Dey, P. M., and Harborne, J. B., Academic Press, New York (1991).
18) Pando, S. C., Oliva, M. L. V., Sampaio, C. A. M., Di Ciero, L., Novello, J. C., and Marangoni, S., Primary sequence determination of a Kunitz inhibitor isolated from *Delonix regia* seeds. *Phytochemistry*, **57**, 625–631 (2001).
19) Koide, T., and Ikenaka, T., Studies on soybean trypsin inhibitor: amino acid sequence of the carboxyl terminal region and the complete amino acid sequence of soybean trypsin inhibitor (Kunitz). *Eur. J. Biochem.*, **32**, 417–431 (1973).
20) do Socorro, M., Cavalcanti, M., Oliva, M. L., Fritz, H., Jochum, M., Mentele, R., Sampaio, M., Coelho, L. C., Batista, I. F., and Sampaio, C. A., Characterization of a Kunitz trypsin inhibitor with one disulfide bridge purified from *Swartzia pickellii*. *Biochem. Biophys. Res. Commun.*, **291**, 635–639 (2002).
21) Yamamoto, M., Hara, S., and Ikenaka, T., Amino acid sequences of two trypsin inhibitors from winged bean seeds (*Psophocarpus tetragonolobus* (L)DC.). *J. Biochem.*, **94**, 849–863 (1983).
22) Batista, I. F. C., Oliva, M. L. V., Araujo, M. S., Sampaio, M. U., Richardson, M., Fritz, H., and Sampaio, C. A. M., Primary structure of a Kunitz-type trypsin inhibitor from *Enterolobium contortisiliquum* seeds. *Phytochemistry*, **41**, 1017–1022 (1996).
23) Di Ciero, L., Oliva, M. L. V., Torquato, R., Kohler, P., Weder, J. K. P., Novello, J. C., Sampaio, C. A. M., Oliveira, B., and Marangoni, S., The complete amino acid sequence of a trypsin inhibitor from *Bauhinia variegata* var. candida seeds. *J. Protein Chem.*, **17**, 827–834 (1998).
24) Wu, H. C., and Lin, J. Y., The complete amino acid sequence of a Kunitz family trypsin inhibitor. *J. Biochem.*, **113**, 258–263 (1993).
25) Kuribara, H., Shindo, Y., Matsuoka, T., Takubo, K., Futo, S., Aoki, N., Hirano, T., Akiyama, H., Goda, Y., Toyoda, M., and Hino, A., Novel reference molecules for quantitation of genetically modified maize and soybean. *J. AOAC Int.*, **85**, 1077–1089 (2002).
26) Sambrook, J., Russell, D. W., "Molecular Cloning: a

Laboratory Manual" 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 7.4–7.8 (2001).

27) Gubler, U., and Hoffman, B. J., A simple and very efficient method for generating cDNA libraries. *Gene*, **25**, 263–269 (1983).

28) Rao, V. R., Corradetti, M. N., Chen, J., Peng, J., Yuan, J., Prestwich, G. D., and Brugge, J., Expression cloning of protein targets for 3-phosphorylated phosphoinositides. *J. Biol. Chem.*, **274**, 37893–37900 (1999).

29) Hung, C. H., Lee, M. C., Lin, M. T., and Lin, J. Y., Cloning and expression of the gene encoding *Acacia confusa* trypsin inhibitor that is active without post-translational proteolysis. *Gene*, **127**, 215–219 (1993).

30) Song, S. I., Kim, C. H., Baek, S. J., and Choi, Y. D., Nucleotide sequences of cDNAs encoding the precursors for soybean (*Glycine max*) trypsin inhibitors (Kunitz type). *Plant Physiol.*, **101**, 1401–1402 (1993).

31) Ashida, Y., Matsushima, A., Tsuru, Y., Hirota, T., and Hirata, T., Isolation and sequencing of a cDNA clone encoding a 20-kDa protein with trypsin inhibitory activity. *Biosci. Biotechnol. Biochem.*, **64**, 1305–1309 (2000).

32) Hammond, R. W., Foard, D. E., and Larkins, B. A., Molecular cloning and analysis of a gene coding for the Bowman-Birk protease inhibitor in soybean. *J. Biol. Chem.*, **259**, 9883–9890 (1984).

33) Gotor, C., Pintor-Toro, J. A., and Romero, L. C., Isolation of a new member of the soybean Kunitz-type proteinase inhibitors. *Plant Physiol.*, **107**, 1015–1016 (1995).